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<b>13. ABSTRACT (Maximum 200 Words)</b> <p>The purpose of the proposed studies is to identify and then target one gene or a small number of genes critical for the development of recurrent growth of CaP. Differential expression analysis, subtractive hybridization and immunohistochemistry were used in the androgen-dependent human prostate cancer CWR22 model to identify 10 genes whose expression might be associated with the onset of CaP recurrence. In the first year of the proposed studies, we visually scored 8 gene proteins in CWR22 tumors on day 120 after castration and identified thio reductase-1, tomor regulin and IGFBP-5 as potential targets; developed a hybrid immunostaining protocol for comparison of expression of antigens in proliferating versus non-proliferating cells; collected the necessary reagents (BrdU-labeled tumors); and demonstrated the feasibility of an antisense approach. In year 2, the grant was suspended after 6 months to allow for transfer to Roswell Park Cancer Institute (RPCI). Quantitative image analysis confirmed that thio reductase-1 expression is higher in proliferating compared to non-proliferating cells in CWR22 tumors that demonstrate growth after castration. Three other nuclear proteins (including Nkx3.1 that was differentially expressed upon visual analysis) revealed no difference using image analysis. Image analysis of seven cytoplasmic proteins is underway using a new image analysis method. In the remaining 1.5 years of the proposed studies, we will complete the image analysis all 10 proteins of interest; examine the genes of interest from the CWR22 model for expression in clinical specimens; and inhibit the expression of the genes of interest using antisense therapy <i>in vitro</i> in CaP cell lines and <i>in vivo</i> in the CWR22 model.</p>				
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## INTRODUCTION

The subject of the proposed studies is the poor performance of androgen deprivation therapy for advanced prostate cancer (CaP). The purpose of the proposed studies is to identify and then target one gene or a small number of genes critical for the development of recurrent growth of CaP. In order to accomplish this goal, we used the human CaP xenograft, CWR22 (Wainstein, 1994). CWR22 retains the biological characteristics exhibited by most human CaP- tumor regression after castration and tumor recurrence approximately 5 months later (recurrent CWR22) (Kim, 2002). Differential expression analysis and subtractive hybridization were used to identify transcripts expressed in intact mice bearing CWR22 tumors and castrated mice bearing recurrent CWR22 tumors but *not in regressed tumors* (Mohler, 2002). Northern and western analyses were used to confirm temporal association with tumor growth before and after castration. Genes identified included 6 androgen-regulated genes [human kallikrein type 2 (hK2), Nkx3.1, insulin-like growth factor binding protein-5 (IGFBP-5),  $\alpha$ -tubulin,  $\alpha$ -enolase and thioredoxin-binding protein 2 (TBP2)] and 4 androgen-*un*regulated genes [tomoregulin, a novel EGF-like molecule, translation elongation factor-1 $\alpha$  (EF-1 $\alpha$ ), Mxi1 and an unknown gene] (Gregory, 2001, Mohler, 2002). Immunohistochemistry was used to recognize small foci of 5-20 proliferating cells that became apparent on day 120 after castration. These foci of proliferating cells immunostained for androgen receptor (AR) and increased levels of prostate specific antigen (PSA), an androgen receptor regulated gene product. The appearance of proliferating tumor cells that expressed AR and PSA 120 days after castration suggests that these cells represent the origin of recurrent CaP. We created tissue microarrays of CWR22 tumors (intact, recurrent and from multiple intervals after castration) that were immunostained with antibodies against tomoregulin, EF-1 $\alpha$  and thioredoxin reductase-1 (TR-1), a member of the TBP2 gene family. All 3 genes were expressed on day 120 after castration, intact CWR22 and recurrent CWR22 but *not in regressed tumors*.

Note: The Year 2 progress report contains brief references to Year 1 work to facilitate reading. Year 2 actually consists of 6 months of funded research activity as a result of transfer of the grant from UNC to RPCI. During the interruption of funding, some work continued on the labor-intensive image analysis. I expect to complete the work of years 1 and 2 in the 6 months of funding that resumed May 1, 2004 so that year 3 is entered with the proposed studies on schedule.

## BODY

### **Aim 1) Complete the identification of genes that are associated with the onset of cell proliferation after castration in the CWR22 model**

Overview: In Aim 1, the expression of the candidate genes will be correlated temporally with cell proliferation and compared directly in Ki-67+ and Ki-67- cells at the precise onset of recurrent proliferation using a full range of immunohistochemical techniques in the CWR22 tumor model.

- Characterize the expression of candidate genes identified using differential expression analysis and subtractive hybridization in the CWR22 model using automated image analysis of immunohistochemical preparations of CWR22 tissue microarrays (months 1-12)

Overview: At the time of proposal submission, the CWR22 tissue microarrays had been immunostained for Ki-67, tomoregulin, EF1 $\alpha$  and thioredoxin reductase-1 (TR-1). In year 1, the tissue microarrays were immunostained for Nkx3.1, IGFBP-5,  $\alpha$ -tubulin, non-neuronal enolase (NNE) and 14-3-3 $\eta$  (Titus, 2004), visual scoring of Ki-67 immunostained adjacent section begun and BrdU-labeled CWR22 tumors obtained. In year 2, the tissue microarrays were immunostained for EF-1 $\alpha$ , hK2, enolase and Mxi-1, visual scoring completed and image analysis begun.

The immunohistochemical evaluation of genes of potential interest continues as described in the master table for the proposed studies. The table describes our progress in securing antibodies, completing immunohistochemical staining of adjacent sections at the onset of cellular proliferation after castration, visual scoring of **foci** of proliferating versus non-proliferating cells in **adjacent sections**, dual labeling using standard

(immunoperoxidase) and fluorescent (anti-BrdU) immunohistochemistry for quantitative image analysis of individual proliferating versus non-proliferating cells **in the same section**.

Adjacent sections immunostained for Ki-67 to identify foci of cellular proliferation and the gene product of interest were scored visually. Five fields of view containing 20 cells were scored for proliferating and non-proliferating areas of each tumor specimen. Each cell was scored from 0 (no immunostaining) to 3 (intense immunostaining) yielding a total score ranging from 0-300 for each tumor area. The tumor visual scores were expressed as the visual score out of 300 and as a fraction of 1 for ease of comparison. In year 1, visual scoring was completed for all but hK2 and EF-1 $\alpha$ . In year 2, visual scoring was completed using adjacent sections (column 5).

Name	Source	Ab	TMA Immuno-stained	Visual Score-Low vs High Prolif Areas in Adjacent Sections	Difference P value	Dual Methods using BrdU (N=nuclear; C=cytoplasmic)	MOD-Low vs High Prolif	Correlation P value
hK2	Mayo	Mouse MAb	Y	0.32 / 0.52		C		
Nkx3.1	Santa Cruz	Goat PAb	Y	0.49 / 0.76	Y	N	0.913 / 0.891	0.15
IGFBP-5	Santa Cruz	Goat PAb	Y	0.24 / 0.52	Y	N	0.938 / 0.937	N
alpha-tubulin	ABCAM	Rat MAb	Y	0.57 / 0.83	Y	C		
NNE	Accurate Chemical	Rabbit MAb	Y	0.62 / 0.60	N	C		
enolase ( $\alpha$ , $\beta$ , and $\gamma$ )	Santa Cruz	Goat PAb	Y			N		
TR-1	Upstate	Rabbit MAb	Y	0.28 / 0.23	N	Y	0.963 / 1.034	0.0033
Tomoregulin	Sakamoto Lab	Mouse MAb	Y	0.68 / 0.62	N	Y	1.235 / 1.226	N
EF-1 $\alpha$	Upstate	Mouse MAb	Y	0.71 / 0.62		C		
MXI-1	BD Pharmigen	Mouse MAb	Y	0.71 / 0.61	N	C		
HSA36	N/A	N/A	N	N/A		C		
14-3-3 $\eta$	Santa Cruz	Goat PAb	Y	0.74 / 0.76		C		

- Confirm the association of candidate genes with the onset of androgen-independent proliferation using immunostaining to compare expression in proliferating (Ki-67 positive) versus non-proliferating (Ki-67 negative) cells in CWR22 tumors 120 days after castration (months 1-12)

In year 1, our image analysis method (Kim, 1999) was modified to allow for measurement of expression of a protein of interest in proliferating (BrdU-labeled) vs. nonproliferating (BrdU-unlabeled) nuclei and CWR22 specimens were BrdU-labeled prior to castration and at various times after castration to generate the biological reagents for these studies. A hybrid peroxidase/fluorescent dual labeling protocol was developed in order to derive robust images for image analysis. Cell proliferation was determined using a fluorescent-labeled BrdU. The proliferating cells were identified using fluorescence that did not interfere with image analysis for the peroxidase-labeled primary.

Our method has proven useful for automated measurement of nuclear proteins in BrdU-labeled vs. BrdU-unlabeled nuclei. We planned to analyze only those proteins of interest that appeared correlated with proliferation upon visual scoring of adjacent sections. Nkx3.1 expression was examined first since it proved most interesting when scored visually (see table). However, there was no difference in Nkx3.1 expression in BrdU-labeled vs. BrdU-unlabeled cells. Only TR-1 revealed a difference among the 4 nuclear proteins. We compared the Ki-67 and BrdU-labeled sections; BrdU-labeled nuclei were relatively rare and visual scoring of

areas that corresponded to areas containing Ki-67 immunostained nuclei were mostly non-proliferating nuclei. Therefore, we decided to image analyze all 11 proteins of interest that required development of an image analysis method for the 7 non-nuclear proteins.

The analysis of cytoplasmic proteins (hk2, alpha tubulin, NNE, EF1 $\alpha$ , Mxi-1, HSA36 and 14-3-3 $\eta$ ) requires an additional mask. A DAPI counterstain was used to identify and remove all nuclei after each cell had been identified as BrdU-labeled or BrdU-unlabeled. Three images (peroxidase, fluorescence and DAPI) were collected from the same location within the sample. The fluorescence and DAPI images were used to create two binary masks. Mask1 was created of the proliferating areas from the fluorescence image by color cube-based segmentation. Mask2 was created of the nuclear regions present in the images from the DAPI image by histogram-based segmentation. Manipulating the peroxidase image using Mask1 created the non-proliferating image. The peroxidase image was added to the negative of Mask1 and the resulting image added to Mask2 to yield the proliferating image. This method has proven effective and will facilitate analysis of the remaining 7 antibodies.

**Aim 2) Correlate the expression of proliferation-associated genes identified in Aim 1 to cell proliferation using clinical specimens of androgen-stimulated and recurrent CaP and serial prostate biopsies performed before and after castration in men with advanced CaP**

**Overview:** The expression of candidate regulatory genes found most promising in Aim 1 will be examined for over-expression in both androgen-stimulated CaP (obtained from radical prostatectomy specimens) and recurrent CaP (obtained from transurethral resection specimens from men with urinary retention due to local CaP recurrence long after androgen deprivation therapy). Over-expressed genes will be examined in 18 series of prostate biopsies obtained before and 1, 4, 7 and every 6 months after castration until recurrence in men with advanced CaP to identify genes that are up-regulated coincidental with the onset of recurrent proliferation.

- Characterize the expression of proliferation-associated genes identified in the CWR22 model in clinical specimens of 50 androgen-dependent and 25 androgen-independent CaPs using automated image analysis of immunohistochemical preparations of tissue microarrays (months 6-18)

The necessary antibodies have been obtained and optimal methods for immunostaining have been developed for 10 gene products of interest. The tissue microarrays are ready for immunostaining and analysis.

- Determine the time of onset of proliferation in the 18 sets of serial prostate biopsies performed before and after castration in men with advanced CaP using automated image analysis of Ki-67-stained tumor specimens (months 1-6)

The first 8 of the 16 invaluable sets of serial prostate biopsies obtained prior to and after castration for advanced prostate cancer were sectioned and H&E stained in year 1. Most of the biopsies did not contain cancer. The possibility that tumor was missed because of the changes that androgen deprivation therapy produces in cancer were discussed with Angelo DeMarzo, M.D., Ph.D. In year 2, racemase and p63 immunostaining was used to highlight the presence of malignant prostatic epithelial cells and basal cells in an attempt to enhance our ability to work with the serial prostate biopsies (Luo 2002; Rubin, 2002). Only 5 of 8 cases had cancer in the enrollment cores that were performed with in one month of the diagnostic biopsies by the same urologist using the same technique. Of these 5 research subjects, only 3 had cancer in their biopsies at the time of progression.

- Characterize the expression of the proliferation-associated genes that appear promising as initiators of recurrent cell proliferation after castration in preliminary study in the CWR22 model and the prostatectomy specimens in the 18 sets of serial prostate biopsies using automated image analysis of immunohistochemical preparations (months 6-18)

Not possible. Instead, we will perform a more detailed analysis using the tissue microarrays described in the previous task.

- Confirm the association of candidate genes with the onset of recurrent cell proliferation after castration using double staining to compare expression in proliferating (Ki-67 positive) versus non-proliferating (Ki-67 negative) cells in serial prostate biopsies that demonstrate the onset of androgen-independent growth (months 12-24)

Not possible. Instead, we will perform a more detailed analysis using the tissue microarrays described in the previous task.

**Aim 3) Determine the effect of altering gene expression upon cell proliferation *in vitro* and serum PSA and tumor growth *in vivo***

Overview: The expression of candidate genes from Aim 2 will be manipulated *in vitro* using antisense probes and the effect upon cell proliferation measured using the androgen-sensitive LNCaP and LAPC-4 cell lines and CWR22 organ culture and the androgen-independent cell lines C4-2 (derived from LNCaP) and CWR-RCaP1 (derived from CWR22). Expression of individual genes will be inhibited *in vivo* to test for effects on tumor growth and serum PSA when antisense oligonucleotides are injected into groups of 12 mice bearing CWR22 tumors 110 days after castration. Effective treatments will be tested more rigorously in groups of 30 tumor-bearing mice.

- Obtain antisense oligonucleotides against best candidate genes from Aims 1 and 2 for use in cell lines and tumors (months 12-24)
- Determine the optimal dose and dosing interval for each antisense oligonucleotide and compare their effects upon cell proliferation using the androgen-sensitive LNCaP and LAPC-4 cell lines, CWR22 organ culture and androgen-independent cell lines C4-2 (derived from LNCaP) and CWR-R1 (derived from CWR22) (months 18-30)
- Determine the optimal dose and dosing interval of antisense oligonucleotides delivered by intraperitoneal injection and their effects on CWR22 tumor cell growth when delivered 110 days after castration (months 18-36)

We have not made any specific progress on Aim 3 since we don't have our gene targets identified yet. However, in year 1, we demonstrated that antisense oligos can be used in prostate cancer cell lines (Mercante, 2002) and that they can be delivered in the nude mouse that constitutes the CWR22 model (Sazani, 2002; Mercante, 2002). In year 2, we have established the cell lines necessary for the proposed studies at RPCI and transferred the CWR22 model to RPCI.

**KEY RESEARCH ACCOMPLISHMENTS**

- Identification of TR-1 as a potential target for preventing the development of CaP recurrence.
- Developed a hybrid immunostaining protocol for comparison of expression of both nuclear and cytoplasmic antigens in proliferating versus non-proliferating cells.

**REPORTABLE OUTCOMES**

**Abstract:**

Titus MA, Gregory CW, Smitherman AB, Subramanian RR, Fu H, Mohler JL, French FS. 14-3-3 $\eta$  protein mediates AR transactivation in recurrent prostate cancer. J Urol 2004; 171 (suppl): 154-155.

## **Manuscripts:**

The abstract above is in final draft as a manuscript for submission to Cancer Research. A manuscript is in first draft that describes the hybrid immunostaining protocol and image analysis methods for comparing nuclear and cytoplasmic protein expression in BrdU-labeled vs BrdU unlabeled cells for submission to Analytical and Quantitative Cytology and Histology. A second manuscript for submission to Prostate will report the visual scoring of adjacent sections immunostained for Ki-67 and the 10 proteins of interest and quantitative image analysis of all 10 proteins of interest in BrdU-labeled vs BrdU-unlabeled cells in the same section at the time of onset of androgen-independent growth in the CWR22 model.

## **Training supported by this award:**

Mark A. Titus, PhD, post-doctoral fellow

## **CONCLUSIONS**

We have made progress in the first 6 months of the second year of this award to identify targets for antisense therapy that can be tested in *in vitro* in CaP cell lines and *in vivo* in the CWR22 model. A hybrid immunostaining protocol and image analysis method has been developed that allows comparison of expression of nuclear and cytoplasmic antigens in proliferating versus non-proliferating cells. This effort will allow a more objective choice of gene target for *in vitro* and *in vivo* studies. Among 4 nuclear proteins of interest, TR-1 has proven a possible candidate. The analysis of the seven cytoplasmic proteins of interest will be completed in the next 6 months. The serial prostate biopsies have proven uninformative and the serial biopsy protocol has been closed at UNC. Potential candidates will be evaluated in the human prostate cancer tissue microarrays and then carried forward to Aim 3 on schedule at the beginning of year 3.

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## APPENDICES

Titus MA, Gregory CW, Smitherman AB, Subramanian RR, Fu H, Mohler JL, French FS. 14-3-3 $\eta$  protein mediates AR transactivation in recurrent prostate cancer. *J Urol* 2004; 171 (suppl): 154-155.

**14-3-3 $\eta$  PROTEIN MEDIATES AR TRANSACTIVATION IN RECURRENT PROSTATE CANCER** *Mark A Titus\*, Christopher W Gregory, Andrew B Smitherman, Chapel Hill, NC; Romesh R Subramanian, Haian Fu, Atlanta, GA; James L Mohler, Buffalo, NY; Frank S French, Chapel Hill, NC*

**INTRODUCTION AND OBJECTIVE:** The androgen receptor (AR) and its primary ligand dihydrotestosterone (DHT) maintain and stimulate growth of prostate cancer (CaP). Androgen deprivation therapy causes regression of CaP through programmed cell death, however, CaP eventually recurs. A role for AR in the growth of recurrent CaP is supported by expression of AR and androgen-regulated genes. 14-3-3 proteins are a family of dimeric  $\alpha$ -helical proteins that interact with a diverse group of intracellular proteins including nuclear receptors. 14-3-3 proteins can bind nuclear receptors to regulate intracellular location and activation. The aim of this study was to characterize the interaction between AR and 14-3-3 $\eta$  and compare expression levels in recurrent CaP, androgen-stimulated (AS) CaP and benign prostate (BP).

**METHODS:** A nickel-charged solid-phase binding experiment was used to measure cell free interaction of 14-3-3 $\eta$  and AR. Coimmunoprecipitation and Western analysis were performed to examine the interaction between 14-3-3 $\eta$  and

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wild-type AR in COS cells or mutant (H874Y) endogenous AR in CWR-R1 recurrent CaP cells. Transient cotransfection assays using CV1 cells were performed to measure AR transactivation by sense or antisense 14-3-3 $\eta$  in presence or absence of DHT. Expression levels of 14-3-3 $\eta$  were compared in clinical specimens of AS-BP, AS-CaP and recurrent CaP, and androgen dependent CWR22 human CaP xenograft tumors using a 14-3-3 $\eta$  specific polyclonal antibody.

**RESULTS:** 14-3-3 $\eta$  protein interacts with mutant (H874Y) AR in the presence or absence of DHT whereas wildtype AR interacts only in the presence of DHT. 14-3-3 $\eta$  sense increased AR transactivation of luciferase reporter activity 4-fold in the presence of 1 nM DHT compared to absence of DHT. In contrast, antisense 14-3-3 $\eta$  decreased luciferase activity below probasin-luciferase and AR alone in the presence of 1.0 nM DHT. Enhancement of wild-type AR transactivation by 14-3-3 $\eta$  was not observed with 10nM progesterone, estradiol or hydroxyflutamide. Finally, the intensity of 14-3-3 $\eta$  immunostaining increased progressively within the cytoplasm of epithelial cells from AS-BP to AS-CaP to recurrent CaP. The CWR22 xenograft model showed androgen regulation of 14-3-3 $\eta$  and similar immunostaining.

**CONCLUSIONS:** Increased expression of 14-3-3 $\eta$  and its interaction with AR in recurrent CaP may activate or stabilize AR during androgen deprivation therapy. Blocking the AR : 14-3-3 $\eta$  interaction may disrupt AR regulation in recurrent CaP and prevent or delay its growth.

**Source of Funding:** HD-07315